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Original Article

Warifteine, an alkaloid of *Cissampelos sympodialis*, modulates allergic profile in a chronic allergic rhinitis model

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ABSTRACT

Cissampelos sympodialis Eichler, Menispermaceae, a Brazilian medicinal plant and its alkaloid warifteine present immunomodulatory activity on asthma experimental model by reducing antigen-specific IgE levels, eosinophil infiltration and lung hyperactivity. Allergic rhinitis is a chronic inflammatory disorder of the nasal tissue that affect the quality of life and it is a risk factor for asthma exacerbation. This study evaluated the effect of inhaled warifteine in an allergic ovalbumin rhinitis model. Inhaled warifteine (2 mg/ml) treatment of ovalbumin-sensitized BALB/c mice significant decreased total and differential number of cells on the nasal cavity and decreased ovalbumin-specific IgE serum levels. Hematoxylin & eosin staining of histological preparations of ovalbumin nasal tissues showed changes such as congestion and a massive cell infiltration in the perivascular and subepithelial regions characterizing the nasal inflammatory process. However, inhaled warifteine or dexamethasone treatment decreased cell infiltration into the perivascular regions and it was observed an intact nasal tissue. Periodic acidic staining of nasal epithelium of ovalbumin animals demonstrated high amount of mucus production by goblet cells and inhaled warifteine or dexamethasone treatment modulated the mucus production. In addition, toluidine blue staining of the nasal epithelium of ovalbumin animals demonstrated an increase of mast cells on the tissue and inhaled warifteine or dexamethasone treatment decreased in average of 1.4 times the number of these cells on the nasal epithelium. Taken these data together we postulate that warifteine, an immunomodulatory alkaloid, can be a medicinal molecule prototype to ameliorate the allergic rhinitis conditions.

Introduction

Medicinal plants and their bioactive components are alternative options to therapies for many diseases. In Brazil, with its enormous biodiversity, the search for new natural products and bioactive molecules is an important goal. *Cissampelos sympodialis* Eichler Menispermaceae, popularly known as "jarrinha" or "milona" is found in Northeastern and Southeast of Brazil and a hot water infusion of its root bark is largely used by indigenous and northeastern population to treat several inflammatory disorders, including asthma, bronchitis, colds and rheumatism (Barbosa-Filho et al., 1997).

Menispermaceae family is well known for producing different types of alkaloids (De Freitas et al., 1996; Barbosa-Filho et al., 2000). In general, alkaloids present a variety of biological activities such as apoptosis and NFkB signaling inhibition in macrophages (The et al., 1990; Thomas et al., 1997), anti-inflammatory and analgesic effects (Costa et al., 2008) and anti-allergic effect (Bezerra-Santos et al., 2012; Vieira et al., 2013; Ribeiro-Filho et al., 2013). Phytochemical analysis of *C. sympodialis* root extracts leads to isolated several alkaloids including warifteine that showed pharmacological effects such as decreasing the allergic process on the experimental model of asthma (Bezerra-Santos et al., 2006).

Warifteine showed to present immunomodulatory effect (Costa et al., 2008) by reducing the plasma levels of total and

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ovalbumin (OVA)-specific IgE, the eosinophilic infiltration into the bronchoalveolar and pleural cavities, the lung hyperreactivity and inducing INF-γ production in OVA sensitized BALB/c mice (Bezerra-Santos et al., 2004). Related to the potential anti-asthmatic effects of *C. sympodialis* and its alkaloid, recent published data showed that inhaled *C. sympodialis* extract or warifteine on OVA-sensitized BALB/c mice down-regulated airway allergic reaction by reducing lung $CD3^+$ T cells and pulmonary hyperactivity (Vieira et al., 2013) on the lung tissue. Therefore, *C. sympodialis* or warifteine effects on allergic rhinitis, a related asthma disease, have not been addressed.

Allergic rhinitis (AR) is defined as an inflammation of the membranes lining the nasal cavity and it is characterized by the following allergic symptoms: sneezing, itching, rhinorrhea, nasal congestion as well as invasion of nasal mucosa by inflammatory cells (Mandhane et al., 2011). A type 2 immune response characterizes AR where the Th2 cell profile predominates as well as eosinophils, mast cells, basophils and macrophages (Palm et al., 2012). The Th2 response is mediated by IL-4, IL-5 and IL-13 and allergic specific immunoglobulin E (IgE) attached to granulocyte cell receptors such as mast cells or circulating basophils. In addition, the AR symptoms occur when the allergen binds to IgE attached to these cells and occurs the release of inflammatory mediator such as histamine, prostaglandins and leukotrienes (Broide et al., 2011).

The incidence of AR has increased in recent years all over the world. Epidemiological surveys indicate that 10–40% of the population, in industrialized and developing countries presents symptoms of rhinitis and its prevalence is about 1.46 billion people worldwide (Véron, 2015). Although AR is not a lethal condition, it can affect quality of life and it is a risk factor for both the development and the exacerbation of asthma (Compalati et al., 2010).

It is well known that antihistamine, anticholinergic agents, intranasal corticosteroids, oral anti leukotrienes and mast cell stabilizers are effective for AR treatment (Stokes et al., 2012) although these treatments can induce adverse side effects including sedation, impaired learning/memory, cardiac arrhythmias and drug tolerance making AR to be less easily control (Mandhane et al., 2011). Therefore, treatments with molecules with potential therapeutic target that causes better effect and less drug tolerance or side effects for AR needs to be explored and developed.

An alternative to ameliorate such condition is medicinal plants and in this scenario the aim of this study was to evaluate the effect of intranasal administration of warifteine, an alkaloid from *C. sympodialis*, as a therapeutic protocol, in an experimental model of allergic rhinitis.

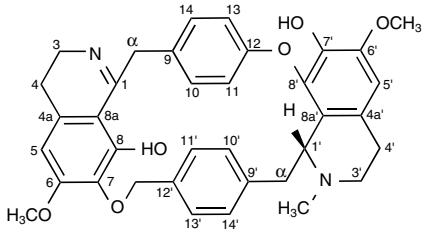
Materials and methods

Animals

Isogenic female BALB/c mice (6–8-week-old), weighing between 25 and 30 g, and female Wistar rats, weighing on an average 200 g, were used in the experiments. The animals were kept in cages at a temperature of 22 ± 2 °C, 12/12-h light–dark cycle with free access to water and a controlled diet, based on pellets, throughout the trial period. The Animal Facility of the Federal University of Paraíba, Brazil supplied the animal strains. Animal manipulation was performed according to animal care guide and the Committee for Experimentation on Animal Research of UFPB (CEUA Nº. 3505/14) approved the experimental protocols. The animals were euthanized with ketamine hydrochloride solution.

Plant material

Cissampelos sympodialis Eichler, Menispermaceae, were obtained from the Botanical Garden of Federal University of Paraíba (voucher specimen Agra 1456). Warifteine (**1**) identification was performed by ^1H and ^{13}C NMR spectral analyses comparing with those published data (Cerqueira-Lima et al., 2010). The alkaloid was quantified in the leaf extract (CsE) by means of High Performance Liquid Chromatography (HPLC) with ultraviolet detection and it was 96.4% pure. The warifteine solution was prepared using 1 mg of the crystal in 50 μl of HCl 1 N and 800 μl of distilled water. The pH was adjusted to 7.0 with NaOH 1 N and the volume was completed to 1000 μl (Costa et al., 2008). The WAR dose (2 mg/ml) and route (inhaled) for the treatment used in this work was based on studies from Vieira et al. (2013).



Treatment

Four groups ($n = 6$) of female BALB/c mice were used in all experimental protocols, and they were divided into saline group (non-sensitized animals), OVA group (OVA-sensitized animals), WAR (**1**) group (OVA-sensitized animals and treated with 2 mg/ml of warifteine), Dexamethasone (DEXA) group (OVA sensitized animals and treated with 2 mg/ml of dexamethasone).

Allergic rhinitis protocol

Mice were ovalbumin sensitized (50 μg OVA emulsified in 5 mg $Al(OH)_3$) on days 0 and 7th by intraperitoneal (*i.p.*) injection. After a week, mice were challenged by nasal instillation of 50 μg OVA on three successive days in a week for three consecutive weeks (between day 14th to day 35th). Two weeks later, mice were re-challenged seven times (from day 49th to day 55th). The OVA, DEXA or WAR groups were treated with inhaled saline, dexamethasone or warifteine 1 h before OVA re-challenge from day 49th to day 55th. Twenty-four hours after the last challenge on day 56th (Fig. 1), the animals were euthanized (Tranquilli et al., 2007) and the nasal cavity lavage fluid (NALF) was collected. The total cell counts were performed in a Neubauer chamber, using an aliquot of diluted samples (1:20) in Turk solution in an optic microscope (40× objective). For differential counts, samples from each NALF of each group were centrifuged on cytospin slides at 1500 rpm for 10 min. The slides were stained with panoptic solution (Panoptic methods/Auto-Hemacolor®) and the cells were quantified in a light microscopy. Each slide was analyzed until the count of 100 cells was reached using oil-immersion objective (Wang et al., 2013).

OVA-specific IgE titer

The serum of each animal from each group was collected from vessels of the brachial plexus. Blood was collected with Pasteur pipette, impregnated with heparin to prevent blood clotting, stored in Eppendorf type tubes inclined at 45 degrees to facilitate clots and then collected the serum that was stored at −20 °C until used. The OVA-specific IgE titer was determined using the passive cutaneous anaphylaxis (PCA) test. The PCA reaction was performed as follows: 50 μl of serum from each animal was injected by dermal route on the shaved back of Wistar rats. After 24 h, the rats were anesthetized with hydrochloride (270 mg/kg *i.p.*), and their tails

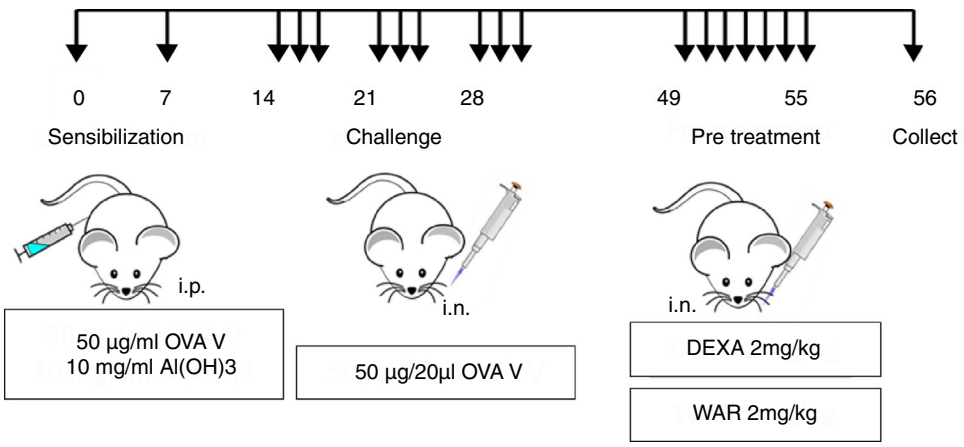


Fig. 1. Allergic rhinitis protocol.

were washed with water and injected, into the vein, 0.5 ml of a solution containing the Evans Blue dye (1% Veteco) and OVA (2 mg/rat, Grade V, Sigma). After 30 min, animals were euthanized, and the OVA specific IgE titer was measured. The highest serum dilution giving a 5 mm diameter flare or bluing reaction was taken as the PCA titer (Holt et al., 1987).

Nasal histology

Twenty-four hours after the last challenge, mice were sacrificed via intramuscular overdose injection of ketamine and xylazine. Their heads were carefully scissored and fixed in tampon formalin for 48 h. Subsequently, the skin was striped, the lower jaw and eyes were removed and the skull was sectioned posteriorly, between the third uppermolar tooth and the posterior opening of the pharyngeal duct (nasopharynx) afterwards, the specimens were immersed in the decalcifying EDTA, dehydrated and embedded in paraffin. Five-micrometer thick sections of paraffin-embedded nasal cavity were stained with hematoxylin–eosin (H&E), periodic acid-Schiff (PAS) and toluidine blue according to standard protocols. The sections were observed for nasal mucosa of allergic inflammation and tissue remodeling under light microscope. Digital photographs were obtained under light microscopy A.T.×100 or ×400 magnifications. For the analysis of inflammatory cell infiltration, H&E stained sections were used (A.T.×100). Mucus secretion from goblet cells of the nasal epithelium was analyzed by the staining of sections with PAS. Goblet cells were recognized by the intense color staining of their mucus content with PAS, and their characteristic distended lateral border and basal nucleus. Toluidine blue was used to observe mast cell in nasal cavity.

Data analysis

The results were analyzed statistically by one-way ANOVA followed by Tukey's test using the Graph Pad Prism software, version 5.0 (Graph Pad Software Inc., San Diego, USA). The results of $p < 0.05$ were considered significant. $^{*}p < 0.05$ were considered significant when OVA group was compared with saline group. $^{+}p < 0.05$ were considered significant when the WAR (2 mg/kg) or DEXA (2 mg/kg) groups were compared with the OVA group. The data of histologic analyses were treated with Kruskal–Wallis test and the parameters considered for the score calculation were cell infiltration, mucus production and numbers of mast cells (Box 1).

Box 1: Score parameters analyzed to nasal histology.

Score	Cell infiltration and mucus
0	Absence of histological changes
1	Mild: less than 25% of the microscopic field
2	Moderate: 25–49% of the microscopic field
3	Marked degree: 50–75% of the microscopic field
4	Very marked degree: over 75% of the microscopic field

Score	Mast cells
0	Less than 5 cells per field
1	5 to 15 cells per microscopic field
2	From 15 to 25 cells per microscopic field
3	From 25 to 35 cells per microscopic field
4	More than 35 cells per microscopic field

Results

Effect of the inhaled warifteine treatment on the NALF

The inhaled warifteine treatment on the experimental allergic rhinitis model caused an inhibition of inflammatory cell migration into the nasal cavity (Fig. 2). The warifteine (WAR) at 2 mg/ml induced a significant decreasing in the total ($p < 0.001$) (0.11 ± 0.05) and differential number of cells (eosinophils (0.008 ± 0.008), neutrophils (0.125 ± 0.06) and mononuclear (0.004 ± 0.003)

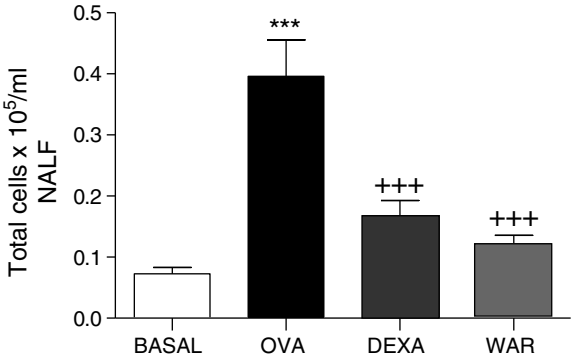


Fig. 2. Effect of the inhaled warifteine (WAR) treatment on the NALF. Cells from the nasal lavage (NALF) of different groups of animals after 24 h of OVA challenge. Results are expressed as the mean ± S.D from six animals. ***Significantly different from saline group ($p \leq 0.001$). $^{+/++/+++}$Significantly different from OVA group ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively). The data are representative of three experiments ($n = 6$).

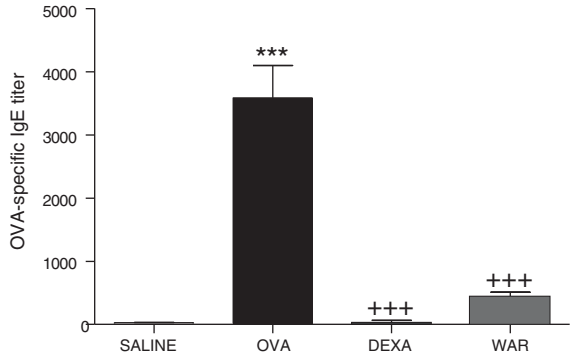


Fig. 3. Effect of the inhaled warifteine on the OVA-specific IgE serum titer BALB/c mice sensitized with ovalbumin (OVA). Sensitized mice were treated with warifteine (WAR) or dexamethasone (DEXA) 1 h before each challenge. $^{+++}$Significantly different from OVA group ($p < 0.001$). The data are representative of three separated experiments ($n = 6$).

when compared to the OVA group (0.39 ± 0.21), (0.133 ± 0.06), (0.262 ± 0.06), (0.034 ± 0.012) respectively. Similar results were observed with dexamethasone (0.16 ± 0.09), (0.008 ± 0.008), (0.142 ± 0.02) and (0.021 ± 0.011) respectively.

Effect of the inhaled warifteine treatment on the OVA-specific-IgE production

Inhaled of WAR induced a decrease ($p < 0.001$) (448 ± 128) of OVA-specific IgE serum titer when compared of OVA animal group (3584 ± 1024) (Fig. 3). The levels of OVA-specific IgE were also significantly reduced in dexamethasone treatment ($p < 0.001$) (48 ± 22.63). This result demonstrated that inhaled WAR has a systemic effect in modulating B cells.

Effect of the inhaled warifteine treatment on the nasal histology

Histological analysis of nasal tissue from non-OVA sensitized mice (salina), OVA-sensitized mice treated with saline (OVA), inhaled WAR or dexamethasone (DEXA) were showed on (Fig. 4A). The H&E staining of the nasal tissue allowed us to observe the morphology of blood vessels, cell infiltration, hypertrophy and hyperplasia of the mucus producing cells and nasal parenchyma. The nasal tissues of OVA group showed pathological changes such as congestion with a massive cell infiltration (asterisk) in the perivascular and subepithelial regions characterizing the inflammatory process as compared with saline group. However, WAR or DEXA groups showed decreasing of cell infiltration (0.8 ± 0.27), (1.1 ± 0.22) into the perivascular regions compared to nasal tissues from the OVA group (4.3 ± 0.27) (Fig. 4B). The PAS staining of nasal tissue from animals of the OVA group demonstrated the presence of mucopolysaccharides with high amount of mucus production by the unicellular exocrine glands (goblet cells) in the epithelial (arrow) as compared with saline group (Fig. 4A). However, the inhaled treatment with WAR (0.9 ± 0.22) or dexamethasone (1.9 ± 0.22) down regulated the mucus production in the nasal cavity when compared with nasal tissue from animals of the OVA group (4.1 ± 0.22) (Fig. 4B). The toluidine blue staining demonstrated high amount of mast cells (arrowhead) in nasal tissue sections of animals from the OVA group (3.2 ± 0.44) as compared to saline group (Fig. 4A). However, WAR (2.4 ± 0.54) or DEXA (2.2 ± 0.44) treatments decreased in about 1.4 times the mast cell number as compared with OVA group (3.2 ± 0.44) (Fig. 4B).

Discussion

In this study, we demonstrate that the inhaled treatment with warifteine (**1**, WAR) on animals from a chronic allergic rhinitis model inhibited inflammatory cell recruitment into the fluid nasal cavity (NALF) and ovalbumin specific IgE serum titer. In addition, the alkaloid treatment decreased the number of mast cells into the adjacent conjunctive nasal tissue as well as mucus production by goblet cells.

Warifteine (1) is an alkaloid of *C. sympodialis*, a medicinal plant used in the Northeastern of Brazil to treat several inflammatory disorders, including asthma and bronchitis. Leave hydroalcoholic extract of the plant has been studied for our group and it showed to present immunomodulatory and anti-inflammatory activities in several experimental models (Vieira et al., 2013).

Allergic rhinitis (AR) is an extremely common disease worldwide affecting 10–50% of world population. Within minutes after a nasal challenge with inhaled allergens, patients present characteristic symptoms such as sneezing, rhinorrhea and nasal congestion. These symptoms are mediated by histamine and other chemical mediators derived from mast cells. Therefore, antigen specific IgE-mediated degranulation of these cells seems to have a crucial contribution to the induction of nasal responses. AR is also characterized by submucosal inflammation associated with massive accumulation of inflammatory cells including eosinophils and T cells. The presence of allergic rhinitis significantly increases the probability of asthma; up to 40% of people with allergic rhinitis have or will have asthma (Bousquet et al., 2008).

Nowadays different classes of drugs are available for the treatment of AR as antihistamines, intranasal glucocorticoids, anti-leukotrienes, decongestants, mast cell stabilizers, and anticholinergic agents (Holgate and Polosa, 2008; Takahashi et al., 2009). These drugs act by different molecular mechanisms to reduce symptoms of AR however, they have many adverse side effects (Holt et al., 1987) Therefore, the use of herbal medicines as an alternative approach for the treatment of allergies has been increasing however, for rhinitis treatment there are few studies with plants and their compounds (Thakare et al., 2013).

In this scenario, we studied the effect of the inhaled warifteine treatment in mice from the chronic allergic rhinitis model. This experimental model is developed by sensitizing BALB/c mice with ovalbumin and challenge, several times, with the allergen to present nasal histological alterations compatible with the human rhinitis. In addition, this experimental model has been used to test drugs by inhaled route due to its low absorption and local effect (Han et al., 2011; Storms et al., 2013).

Inhaled WAR treatment decreased the migration of inflammatory cells such as eosinophils to the nasal lavage fluid (NALF) of animals from a chronic allergic rhinitis model. These cells are involved on the pathogenesis of allergic rhinitis and they migrate to the nasal tissue due to the type 2 immune response involving the Th2 cell activation and production of cytokines such as IL-4, IL-5 and IL-13 (Barnes, 1999). IL-5 is responsible for the eosinopoiesis during the allergic process (Bentley et al., 1993). Therefore, this result indicates that the alkaloid may be modulating the type 2 immune response on this experimental model.

Previous studies of our group demonstrated that oral treatment with WAR has a profound effect on eosinophil function, decreasing eotaxin levels, cysteinyl-leukotriene generation and cytoplasmic lipid body formation (Bezerra-Santos et al., 2006). In addition, Vieira et al. (2013) demonstrated that inhaled administration of *C. sympodialis* leave hydroalcoholic extract alleviated airway allergic reactions by reducing $TCD3^{+}$ cells on the lungs.

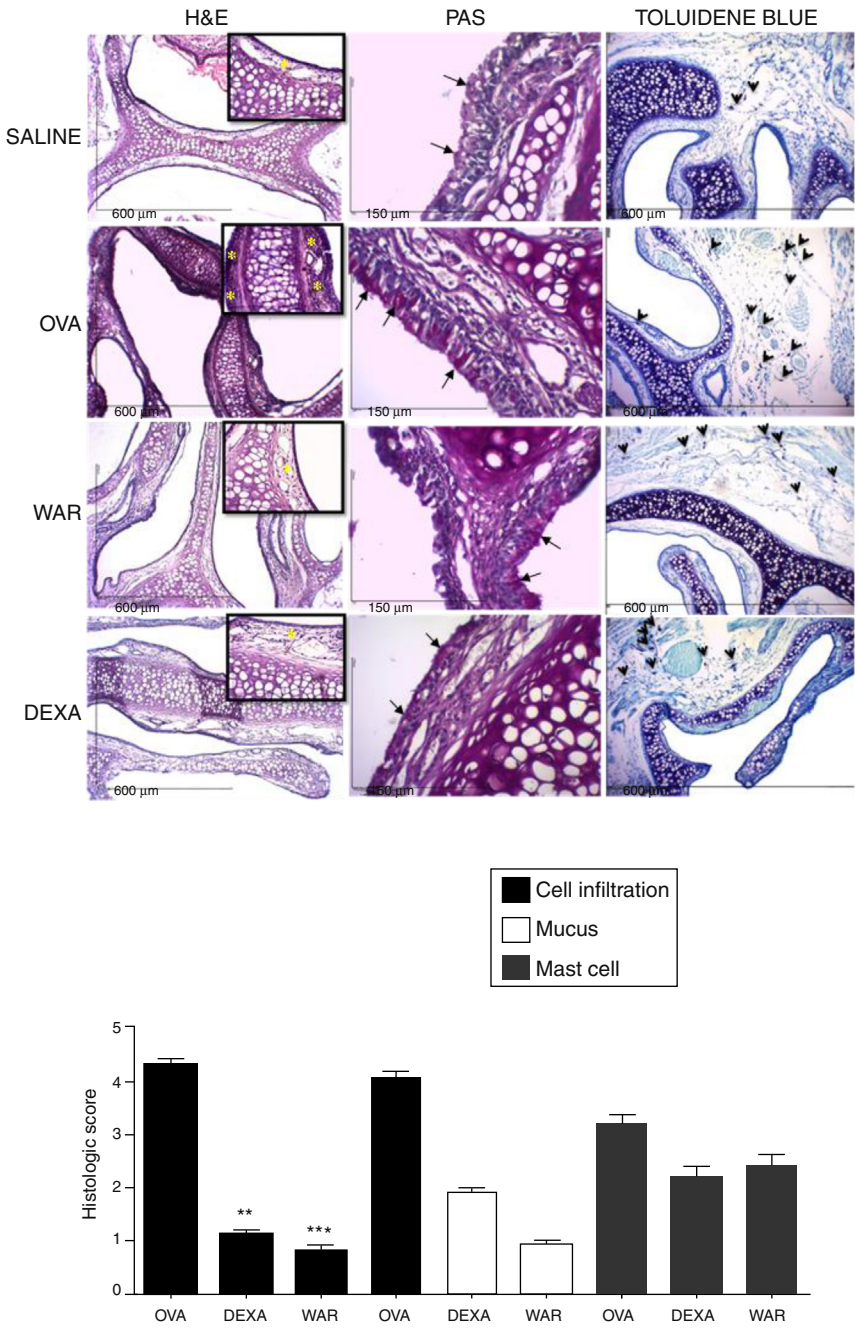


Fig. 4. (A) Photomicrograph representative of the upper airways-nasal cavity stained with HE AT 100×, PAS AT 400× and toluidine blue AT 100×. For the histological analysis of four nasal sections of each animal group, SALINE; OVA; DEXA; WAR. (B) Histologic score of the upper airways nasal cavity to quantify cell infiltration, mucus and mast cell. Histological analysis of four nasal sections of each animal group OVA; DEXA; WAR. Results are expressed as the mean ± S.D from four animals. */**/***Significantly different from OVA group ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively).

Further, inhaled WAR also had a systemic effect decreasing OVA-specific IgE serum titer of animals with allergic rhinitis showing down regulation on antigen specific B cells. Rocha et al. (2010) demonstrated the oral treatment with WAR decreased the production of IgM and IgG in several *in vitro* and *in vivo* experimental models reassuring the immunomodulatory property of WAR on B cells (Rocha et al., 2010).

One of the main effector cell on allergic processes is mast cell, which is dependent of IgE-mediated activation pathway (Galli and Tsai, 2012). When a person is exposed to an allergen that was sensitized, cross-linking between the allergen and IgE bounded to mast cell receptors results the release of several mediators such as histamine, prostaglandin D2, and cysteinyl leukotrienes with nasal symptoms. During the next hours, through a complex interaction of mast cells, epithelial cells, dendritic cells, T cells, innate lymphoid cells, eosinophils, and basophils occurs the release of a wide array of chemokines and cytokines that up regulate the nasal symptoms that persist for hours after allergen exposure. The nasal mucosa becomes more reactive to the precipitating allergen (priming) as well as to other allergens and to nonallergenic stimuli, such as strong odors and other irritants (Sin and Togias, 2011). Therefore, drugs that stabilized or decreased the number of mast cells into the nasal tissue would improve the rhinitis symptoms.

In this study, we demonstrated that WAR reduced the production of IgE, one of the immune molecule responsible for mast cell degranulation and also, slight, reduced the number of mast cells in conjunctive tissue next to the nasal cavity of animals with rhinitis. Therefore, WAR treatment decreased mucus production in nasal proximal squamous epithelium, resulting in decreasing of hyperplasia and hypertrophy of goblet cells.

Previous studies where it was used leave extract of *C. sympodialis* by inhaled route to treat animals with allergic asthma showed reduction of inflammatory cell infiltration as well as mucus production by lung tissue histological analysis (Vieira et al., 2013). The authors of this study suggested that WAR, as a major compound of extract, had an important role in modulating the inflammatory reaction on the lung tissue of these animals.

In summary, inhaled WAR exerts anti-allergic effects in OVA-induced allergic rhinitis by decreasing OVA-specific IgE serum level and ameliorating nasal histopathological aspect such as decreasing the number of mast cells and mucus production. However, molecular mechanisms involved in theses anti allergic process need to be clarified and further studies is under investigations.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

PTB and MRP designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. GCV, FAAFG, RFP and LKDPF were responsible for execution of the project and conducted data analysis and drafted the paper. JMBF gave warifteine for the study. All the authors reviewed and approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest

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